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Award Number: W81XWH-12-1-0003

TITLE: Induced Accelerated Aging in Induced Pluripotent Stem Cell Lines from Patients with Parkinson's Disease

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REPORT DATE: November, 2012

TYPE OF REPORT: Annual Technical Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
November 2012	Annual	14November2011 – 13November2012

4. TITLE AND SUBTITLE

Induced Accelerated Aging in Induced Pluripotent Stem Cell Lines from Patients with Parkinson's Disease

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0003

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Parkinson's Institute

Sunnyvale, CA 94085

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Background: iPS reprogramming to model 'disease-in-a-dish has become an attractive approach to study disease mechanisms. However, a frank pathophysiological phenotype in iPSC-derived PD neurons remains to be shown.
Objective/Hypothesis: The objective of this proposal is to accelerate the aging process of iPS-derived dopaminergic neurons with the goal of reproducing a Parkinson's disease (PD) specific phenotype in vitro.
Specific Aim 1.
To generate vector constructs and to establish Dox-inducible iPSC lines that express the Hutchinson-Gilford progeria (HGPS) gene (mutant lamin A with an in-frame loss of 150 nucleotides) and differentiate them into dopaminergic neurons.
Deliverable of this aim is the introduction of mutant lamin A in iPSC lines and optimization of inducible expression of mutant lamin A in iPSCs and during the differentiation into dopaminergic neurons.
Specific Aim 2.
To test whether iPSC-derived dopaminergic neurons transduced with mutant lamin A show changes in age-regulated genes at the mRNA and protein levels at different time-points during differentiation, thus exhibiting an accelerated aging.
Deliverable of this aim is the assessment of specific age-related aging pattern of gene and protein expression in induced mutant lamin A modified iPSC-derived neurons.
Specific Aim 3.
To test whether induced mutant lamin A cell lines differentiated into dopaminergic neurons exhibit hallmark pathology of PD, such as protein aggregation of alpha-synuclein, posttranslational modification, and signs of mitochondrial pathology.
Deliverable of this aim is the assessment of the pathological PD-related phenotype in induced mutant lamin A modified iPSC-derived neurons.
Study Design: This is an in vitro study of patient-specific iPSC-derived dopaminergic neurons in which truncated lamin A will be introduced to study cellular phenotypes under the hypothesis that cells under expression of lamin A will age faster than untreated cells.
Relevance: Creation of iPSC lines from patients with PD that develop a disease phenotype would revolutionize research in PD and could produce more predictive disease models to enable the advancement of better candidates into clinical testing. If successful, the impact of this research project on the iPSC field could be enormous. It could remove one of the remaining roadblocks to using iPSC model for PD for the study of disease mechanism and drug development, which could bring us closer to finding the cause and cure for PD.

15. SUBJECT TERMS
Parkinson's disease, induced pluripotent stem cells, cellular model, accelerated aging, lamin A, progerin

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU		19b. TELEPHONE NUMBER *(include area code)*

Annual Report

Principal Investigator: Birgitt Schuele, MD

Award No.: W81XWH-12-1-0003

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Table of contents:

Introduction	p.3
Body	p.3-6
Key Research Accomplishments	p.6
Reportable Outcomes	p.6
Conclusion	p.6
References	p.7
Appendices	p.8

Introduction

The *objective of this study* was to introduce the truncated lamin A gene, progerin, into patient-derived induced pluripotent stem cells using a doxycycline (Dox)-inducible expression system and differentiated them into dopaminergic neurons. The *hypothesis* is that mutant lamin A, which is causing clinically an premature aging syndrome called Hutchinson-Gilford progeria, will accelerate the aging process in the culture dish and facilitate changes related to a PD phenotype.

The *underlying goal of this proposal* is to optimize the current cellular iPSC model of PD aimed at reproducing a pathological phenotype in a culture dish and establishing a 'disease-in-a-dish' model for PD.

By using the progeria gene to experimentally age the iPS-derived DA neurons, we hypothesize that these cells age faster and therefore adding this important 'risk factor for PD' to the culture system presumably without interfering with the known disease related mechanisms of PD.

Body

Generation of the drug-inducible construct using Clontech Retro-X Tet-On advanced vector

We obtained different plasmids from Addgene (www.addgene.org) containing different forms of progerin: pBABE-puro-GFP-progerin (Cat. No. 17663) and pEGFP-D50 lamin A (Cat. No. 17653). The plasmids were transformed into E.coli and plasmid DNA was extracted and the gene insert was sequenced including boundaries to ensure the correct nucleotide sequence and restriction sites of the plasmid. The progerin gene insert was packaged in VSV-G pseudotyped retrovirus. We have the following constructs to enable creation of drug-inducible iPSC lines and to be evaluated in iPSCs and differentiated dopaminergic neurons:

1. pRetoX-Tet-on
2. pRetroX-Tight-Pur-GFP-Progerin
3. pRetroX-Tight-Pur-Progerin

Establishing stable iPSC lines with drug-inducible progerin construct

iPSC lines obtained from a Parkinson's disease (PD) patient with a gene triplication in the alpha-synuclein gene with early-onset progressive autosomal-dominant PD and one from control were transfected with pRetroX-Tet-on to establish stable iPS lines expressing the transactivator rtTA advanced protein. The clones obtained after infection were screened for the successful expression of the rtTA advanced protein through Western blot analysis using the TetR monoclonal antibody. After selection of clones that are positive for inducibility, the clones are being currently infected with pRetroX-Tight-Pur-Progerin.

The iPS cells were dissociated into single cells and then transduced with the pRetroX-Tet-on expressing the rtTA protein. After transduction the cells were grown in selection media containing the antibiotic G418 (Figure 1).

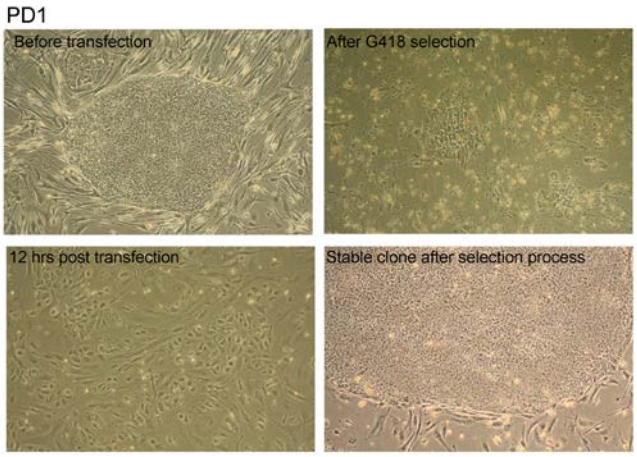

Figure 1. Transduction process and example of stable rtTA expressing line.

We have 12-15 clones picked from each iPSC line after transfection with pRetroX-Tet On. 6 and 7 clones each for each iPSC clonal lines (PD1 and PD2) from patient with triplication in the SNCA gene survived after multiple passages. The main problem was the slow growth and therefore difficult expansion. 12 clones for the control line (CL1) survived after multiple passages. All clonal lines grew fast and are easy to expand in culture.

We are now in the process of growing the cells for the pRetroX-Tight-Pur-Progerin transfection and the pRetroX-Tight-Pur-GFP-Progerin transfection.

We have encountered several issues that delayed the project: 1. the timeline estimations of the original grant were based on immortalized cancer cell lines that have a faster doubling time compared to stem cells which show a much slower growth. 2. human patient-derived stem cells are more vulnerable to virus and conditions needed to be optimized including the use of antibiotic resistant feeder cells that support the growth of the cells.

Co-transfection of iPS lines with Tet-on and progerin:

Besides the outlined consecutive transduction of the transactivator and then the progerin gene, we are also currently performing a co-transfection with pRetroX-Tet-on and pRetroX-Tight-Pur-GFP-Progerin to establish double-stable iPS lines expressing the rtTA and GFP-Progerin proteins.

The project received a nine month no cost extension to complete the milestones. We are confident that we will complete the study in this time period.

Advancement of Neuronal differentiation

We had established a neuronal differentiation protocol using embryoid body differentiation to obtain neural progenitor cells (NPC) (Mak et al., 2012) using a combination of two small molecules (SMAD inhibitors). This has been a cumbersome protocol which was taking up to 8 weeks to derive NPCs.

We are now in the process to establish a new protocol without embryoid body differentiation to derive NPCs within 10-14 days (Figure 2). iPS cells are maintained in Neural induction media for 7 days, then dissociated and replated onto Matrigel. At passage 2, the cells were stained for the neuroprecursor marker nestin (Figure 2, panel 6, red) and showed a very high conversion rate. This allows us to faster differentiate the clones once they are derived and will save time at Milestone 4 of the proposal.

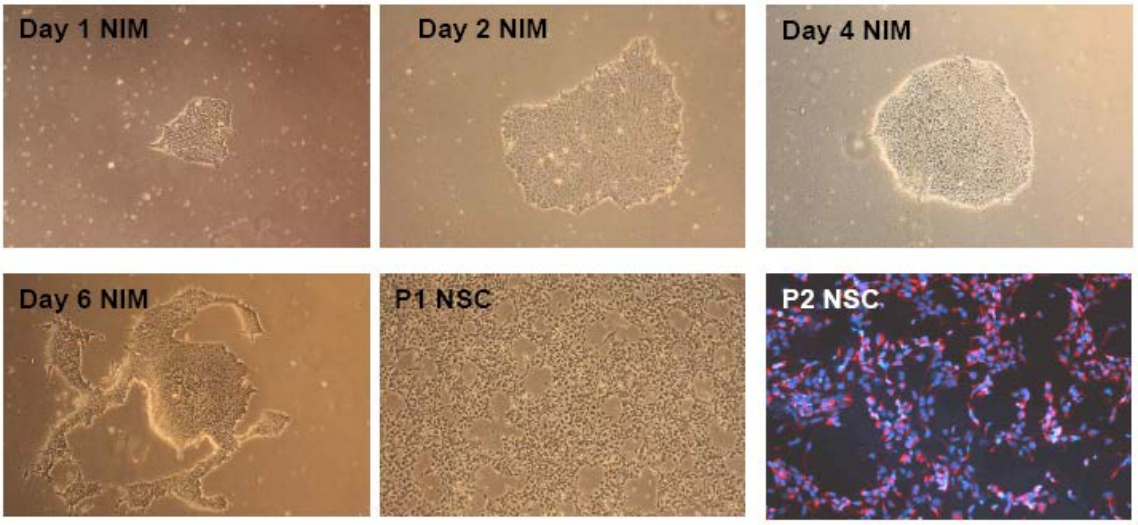

Figure 2: Advanced neuronal induction with direct differentiation under feeder-free defined conditions

Establishment of aging markers

One goal of the proposal as part of Milestone 5, we want to define an age-related phenotype. One established marker is the expression of the tau isoform 3R and 4R (Iovino et al., 2010). We are currently testing the expression in naïve or untransfected stem cell cultures in the alpha-synuclein triplication cell lines compared to the control lines and will have the assay optimized once the progerin lines are derived.

Key Research Accomplishments:

- *Milestone 1* (month 1-2): generation of the drug-inducible construct using Clontech Retro-X Tet-On advanced vector **COMPLETED**
- *Milestone 2* (month 2-3): establishing stable iPSC clones with drug-inducible progerin construct **IN PROGRESS**
- *Milestone 3* (month 4-6): differentiation into DA neurons for all generated lines **OPTIMIZATION PHASE**
- *Milestones 4* (month 6-12): examination of DA-phenotype, age-related phenotype, and pathological phenotype related to PD **NOT STARTED**

Reportable Outcomes:

At this point, no publications or posters have been derived from the grant, however, an abstract is planned for 2013 annual meeting of Neuroscience Society (SFN).

Conclusions:

Creation of iPSC lines from patients with PD that develop a disease phenotype would revolutionize research in PD and could produce more predictive disease models to enable the advancement of better candidates into clinical testing.

Even though there has been a delay in the progress due to the difficulties of utilizing human patient-derived stem cells, we think that there is a great value in the derivation of these cell lines.

It will also allow us to study in more detail the recent findings of nuclear envelope changes in another genetic PD syndrome, LRRK2 parkinsonism (Liu et al., 2012).

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Liu, G.H., Qu, J., Suzuki, K., Nivet, E., Li, M., Montserrat, N., Yi, F., Xu, X., Ruiz, S., Zhang, W., *et al.* (2012). Progressive degeneration of human neural stem cells caused by pathogenic LRRK2. Nature *491*, 603-607.

Mak, S.K., Huang, A.Y., Iranmanesh, S., Vangipuram, M., Sundararajan, R., Nguyen, L., Langston, J.W., and Schüle, B. (2012). Small molecules greatly improve conversion of human induced pluripotent stem cells to the neuronal lineage. Stem Cells International.

Appendices

Protocol for development of double stable cell line for progerin

Plasmids	pRetro-X Tet-On + pRetro-X Tight-Pur-GFP progerin
Cell densities tested	200K and 500K cells per well of a 6-well
Lines tested	HUF4 c17 p21, HUF4 c2 p21, 1827 A34-1
Mode	Infection in suspension

•All measurements are for 2mL hESC media volume

• Treat iPS cells on Matrigel with 10uM Rock Inhibitor (20uL of 1mM stock) the day prior to viral infection

• Individualize cells by treatment with Accutase (2mL per 6-well well for 10mins) and count

• Incubate appropriate number of iPS cells with the virus (assuming, 1uL of $1x10^9$ = 1 MOI, add 3uL each of pRetro-X Tet-On and pRetro-X Tight-Pur-GFP progerin virus), 4ug/mL polybrene (0.8uL of 1000ug/mL) and 10uM Rock Inhibitor (20uL of 1mM stock) in suspension (Use 6-well plate with Matrigel on the rocker) for 1 - 2 hour in the incubator

• Centrifuge cells gently (500 x g for 5 mins) and remove supernatant thereby removing the excess virus

• Plate cells on MEFs with 10uM Rock Inhibitor (20uL of 1mM stock)

• Allow cells to grow for 48 hours and treat with 200ug/mL G418 (Clontech # 631307) for 7-14 days, using the optimal concentration

• Change media every day

• Colonies with ~100 cells can be picked and plated on Matrigel/MEFs in a 12- well plate

• Expansion